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On the Mode of Action of Thrombin-mediated Angiogenesis: Functional Characterization of RGD Sequence within Thrombin

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S u m m a r y. We previously presented evidence that thrombin interacts with αvβ3 integrin in human um bilical vein endothelial cells (HUVEC) and that this interaction contributes to activation of angiogenesis by thrombin (1). In the present study we confirm that, like thrombin, immobilized TP508, a synthetic thrombin peptide that contains the thrombin RGD sequence (2), supported integrin αvβ3-dependent endothelial cell (EC) attachment and aptotactic migration. These effects required intact the RGD sequence and stimulated signal transduction pathways characteristic of integrin activation. We found that soluble TP508 acts as an antagonist of thrombin at the level of MAP kinases activation. These results raise the importance of RGD sequence within the thrombin molecule and establish the pivotal role of integrin αvβ3 in mediating thrombin-induced angiogenesis.

INTRODUCTION

Thrombin, known for its direct role in clot formation, has a variety of cellular effects relevant to tumor growth and metastasis (3). It regulates cellular behaviour by activating protease-activated receptors (4). Many of the effects of thrombin in cancer may be explained by our finding that thrombin promotes angiogenesis (5). Angio-genesis involves several events and we have shown that thrombin contributes to each of these events.

Recently we showed that EC attach to thrombin via αvβ3 integrin, which is up-regulated by thrombin molecule. In the present study we used the synthetic thrombin peptide TP508 that contains the RGD motif, in order to elucidate the role of RGD sequence within thrombin in thrombin-promoted angiogenesis.

METHODS

Cell attachment assay.- Forty eight-well plates were coated overnight in room temperature with the indicated concentrations of TP508 or thrombin. The wells were blocked for 60 min with 2% BSA in PBS at 37 °C. Cells were then incubated in the presence or absence of increased concentration of TP508, the specific αvβ3-integrin an-tagonist cyclic peptide EMD121974 (cRGD, Merck), the inactive cyclic peptide EMD135981 (cRAD, Merck) or the mouse anti-αvβ3 (LM609) antibody (Chemicon) for 15 min at 37 °C. Adhered cells were fixed, stained and the average area covered by them was measured in triplicate wells with a computerized digital image analyzer.

Western blot analysis.- 60mm plates were coated overnight at room temperature with the indicated concentration of poly-L-lysine, or TP508, or vitronectin and blocked with 2% BSA in PBS for 1 hour at 37 °C. Cell suspension (5x105 cells/plate) was then added to the plates and incubated at 37 °C for 30 min. Adherent cells were lysed of SDS-polyacrylamide gel sample buffer. Cell lysates were boiled and electrophoresed (10% gel) and transferred to PVDF membrane using stardard procedures. The membranes were blocked in 5% instant nonfat milk powder in PBS and incubated overnight at 4 °C with anti-phospho-p42/44 MAPK or anti-p42/44 MAPK antibodies. Membranes were then probed with HRP conjugated

secondary antibodies for 1-2 hours at room temperature and proteins were visualized by chemiluminescent detection. For analysis of MAP kinases activation triggered by soluble proteins or peptides, HUVECs were cultured in 35mm plates. After reaching confluency, cells were growth factor-starved by incubation in M199 medium containing 0,5% FBS for 24 hours. Cells were then stimulated for 10 min with the indicated proteins or peptides. Subsequently, cells were lysed and the procedure was followed as mentioned above.

RESULTS

Thrombin peptide TP508, like thrombin, promotes endothelial cell attachment and migration.

We have previously shown that immobilized thrombin promotes EC attachment and migration in an αvβ3- and RGD-dependent manner (1). In order to determine whether TP508 sequence within thrombin is responsible for these cellular effects, we examined the ability of HUVECs to be attached and migrate on TP508 peptide. As shown in Figure 1, TP508 promotes EC attachment and this effect is abolished by the presence of αvβ3 integrin antibody (LM609) or the cRGD peptide, an antagonist of αvβ3 integrin. The inactive cRAD peptide had no effect. TP508(RAD), chemically modified TP508 peptide that contains a RAD motif instead of RGD, did not support EC attachment and migration.

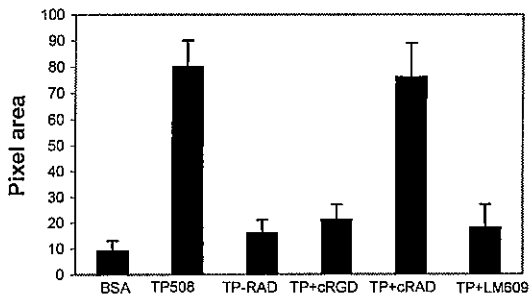

Figure 1. Immobilized thrombin peptide TP508 on plastic surface promotes endothelial cell attachment. BSA: bovine serum albumin, TP-RAD: modified TP508 peptide that has RAD instead of RGD, cRGD: antagonist of αvβ3 integrin, cRAD: inactive cRGD, LM609: antibody for αvβ3 integrin.

Adhesion of endothelial cells to TP508 peptide induces the activation of FAK and MAPK

Activation of integrins after cell adhesion to ECM proteins leads to stimulation of numerous intracellular signal tranduction pathways including focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK). We evaluated the phosphorylation status of these molecules after exposure of EC to thrombin, TP508 or vitronectin, the prototypic ligand for αvβ3 integrin. We found that EC attached to TP508 featured phosphorylated FAK and MAK whereas these molecules were not activated in suspended cells.

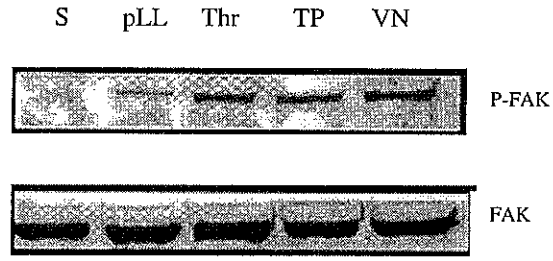

Figure 2. The attachment of endothelial cells on TP508 coated surface causes phosphorylation of FAK. S: cells in suspension, pLL: polylysine, Thr: thrombin. VN: vitronectin

Soluble TP508 peptide inhibits thrombin-induced MAPK activation

We have shown that soluble TP508 antagonizes thrombin-induced cellular effects including MMP-2 activation, VEGF upregulation and prostacyclin release. Thrombin activates a signaling pathway that involves key enzymes such as phospholipase C, MAPK and phospholipase A_2. We found that soluble TP508, but not TP508(RAD), antagonizes in an RGD-dependent manner the cellular effects of thrombin by inhibiting the thrombin-induced phosphorylation of MAPK and PGI_2 release whereas it did not change the elevated levels of inositol phosphates caused by thrombin.

CONCLUSIONS

These results suggest that the RGD motif in TP508 peptide plays an essential role in EC adhesion and migration. TP508 within the thrombin molecule is probably responsible for the ability of immobilized thrombin to cause attachment and migration of EC. It is suggested that these effects are mediated through the αvβ3 integrin on the surface of EC and they involve the RGD sequence of the thrombin molecule. Taken together, these results establish the pivotal role of integrin αvβ3 in mediating thrombin-promoted angiogenesis and suggest a potential mechanism for thrombin effects in EC.

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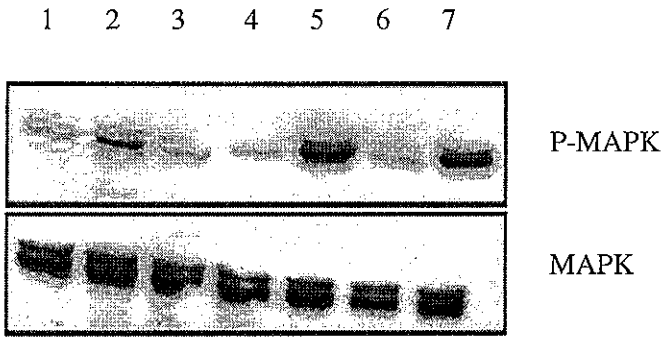

Figure 3. Soluble thrombin peptide TP508 but not scrambled peptide (scrb) activates MAPK, like thrombin, in endothelial cells. 1: Control; 2: thr; 3: TP508 10 μM; 4: TP508 100 μM; 5: Thr+TP508 10 μM; 6: Thr+TP508 100 μM; 7: Thr+scrb TP508